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ORIGINAL ARTICLE

Synergistic effects of verapamil on pingyangmycin-induced cytotoxicity and apoptosis in KB cells

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OBJECTIVES: Previous studies have shown that pingyangmycin (PYM; bleomycin A5) can induce two distinct modes of cell death (necrosis, apoptosis). At high concentrations, PYM can be considered as an apoptosis mimetic drug. In this study, we explored the possibility that the membrane-modifying agent verapamil might affect the transport function of PYM through the plasma membrane, resulting in inducing apoptosis of tumor cells at low concentration of PYM.

METHODS: Cytotoxicity, flow cytometry and DNA fragmentation assays were used to detect the interaction of verapamil and PYM in human oral carcinoma cell line KB cells.

RESULTS: Our results indicated that verapamil can enhance the cytotoxicity of PYM against KB cells with the non-toxic doses ($P < 0.05$). The cell viability at a concentration of 500 μg ml^{-1} of PYM was 35 ± 2% compared with control and 10 μg ml^{-1} verapamil decreased the cell viability lower to 28 ± 1%. In addition, because of the synergistic effect of verapamil, KB cells apoptosis was found to be induced when treated with a lower concentration of PYM (50 μg ml^{-1}) for 24 h by flow cytometry and DNA fragmentation assays.

CONCLUSIONS: Verapamil was found to enhance PYM-induced cytotoxicity and apoptosis in KB cells. The responsiveness of PYM might be explained by the effective accumulation of PYM by verapamil in KB cells mediated by the inhibition of PYM efflux function of the cells.

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Introduction

Chemotherapy for malignant tumors has been advanced by the development of a variety of effective anticancer agents. Understanding the cellular processes involved and the molecular mechanisms responsible for the cell death induced by anticancer drugs may aid in the development of more effective and tumor-selective chemotherapy (Skipper *et al*, 1978; Nishimura *et al*, 1979). There were two major morphologically and biochemically distinct processes whereby death occurred in eukaryotic cells: apoptosis and necrosis (Wyllie *et al*, 1980). The mode of cell death has received much attention, as it has been found that many chemotherapeutic drugs induce apoptosis in a variety of tumor cell lines (Hickman, 1992). However, the ability of these drugs to induce cell death is often limited by the development of drug resistance in the malignant cells. Earlier experiments have shown that the most important mechanism of drug resistance was attributed to the enhanced drug efflux function of resistant tumor cells (Nishimura *et al*, 1979; Tsuruo *et al*, 1983a). These observations suggest that by controlling the drug efflux function of tumor cells appropriately, anticancer agents could be expected to exert a therapeutic effect against resistant cells.

The antitumor antibiotic pingyangmycin (PYM; bleomycin A5) was screened out and developed from many components of bleomycin produced by *Streptomyces pingyangensisn*. PYM has a chemical structure similar to that of bleomycin, with the only difference being moiety of the terminal amine (Umezawa *et al*, 1966). The spermidine derivative (A5) is the major component of PYM. In China, PYM is extensively used in chemotherapy for the treatment of squamous cell carcinoma, malignant lymphoma, Hodgkin's disease, and lymphangioma (Wang, 1989; Zhong *et al*, 1998). Previous studies have shown that PYM can induce two distinct modes of cell death – apoptosis and necrosis (Tai *et al*, 2000, 2003). Cells become enlarged, multinucleated, and display an arrest in G2-M phase of the cell cycle in the presence of low concentrations of PYM (5 and 50 μg ml^{-1}). In contrast, cells exhibit the morphological and biochemical changes associated with apoptosis when treated with high concentrations of PYM (500 μg ml^{-1}, 5 mg ml^{-1}). However, in order to induce cell apoptosis, the above concentrations of PYM present in the culture medium is too high for clinical use.

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Verapamil has been commonly used to treat arrhythmia and angina pectoris (Fleckenstein, 1977). The primary target of verapamil is presumed to be cell membranes because of the lipophilic side chain (Bayer *et al*, 1975). A well-known action of verapamil is its inhibition of the slow channel of Ca^{++} transport across the cell membrane (Langer *et al*, 1975). Previous reports have described that verapamil can enhance the cytotoxicity of many antitumor agents against tumor cells *in vitro* through an effective accumulation of these drugs in tumor cells. Enhanced accumulation of antitumor agents was attained by the inhibition of efflux function of the cells of these drugs (Skovsgaard, 1978a,b; Inaba *et al*, 1979).

In this study, we explored the possibility that the membrane-modifying agent might affect the transport function of PYM through the plasma membrane, thereby inducing apoptosis of tumor cells at lower concentrations of PYM. Thus, cytotoxicity, flow cytometry and DNA fragmentation assays were used to evaluate the effects of verapamil on PYM against human oral carcinoma cell line KB cells.

Materials and methods

Chemicals and materials

Verapamil and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Pingyangmycin hydrochloride was purchased from Taihe Pharmaceutical Co. (Tianjin, China); appropriate doses were prepared in Dulbecco's modified Eagle medium (DMEM). All culture materials were obtained from GIBCO (Grand Island, NY, USA).

Cell Culture

KB cells, a human oral squamous cell carcinoma cell line (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% fetal calf serum, 100 μg ml^{-1} of streptomycin, 100 mg ml^{-1} of penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every third day.

Cytotoxicity assay

The MTT assay is a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. In brief, MTT solution was prepared as 1 mg ml^{-1} in complete medium just before use. KB cells were diluted in fresh complete medium and seeded in 96-well plates (1×10^4 cells/well). After overnight attachment, cells were divided two groups. The cells of group 1 were treated with various concentrations of verapamil (5, 10, 20, 40, 80 μg ml^{-1}) for 24 h, and the other were treated with different doses of PYM (0.5, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) with or without verapamil (5, 10 μg ml^{-1}) for 24 h. MTT dye (50 μl/well) was added to each well and the plates were incubated in a CO_2 incubator for 4 h. Optical density was determined by eluting the dye with dimethyl sulfoxide, and the spectrophotometric absorbance was measured at 550 nm using an ELISA reader (Σ960; Meterteck, Tokyo, Japan).

Flow cytometry

Flow cytometry was performed on cells stained with propidium iodine as detailed previously (Telford *et al*, 1991). KB cells were plated 2×10^5 cells per well into 6-well plates. After overnight attachment, they were treated with different doses of PYM (5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) with or without verapamil (5, 10 μg ml^{-1}) for 24 h. The floating cells in the medium were then combined with attached cells harvested by trypsinization. Cells were washed with cold phosphate-buffered saline (PBS) and fixed in 80% ethanol in PBS at −20°C. After 12 h, fixed cells were pelleted, gently resuspended in cold PBS and supplemented with 0.5 mg ml^{-1} propidium iodide plus 50 μg ml^{-1} RNase A. Samples were then incubated at 37°C for 30 min, stored in the dark at 4°C, and analyzed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

DNA fragmentation

Fragmentation of chromosomal DNA was analyzed by an agarose gel electrophoresis (Herrmann *et al*, 1994). Exponentially growing cells were seeded in 6-well plates (2×10^5 cells/well). After 24 h, cells were treated with different concentrations of PYM (5 μg ml^{-1} to 5 mg ml^{-1}) with or without verapamil (5, 10 μg ml^{-1}) for 24 h. The cells were harvested and washed in cold PBS and pelleted by centrifugation. Cell pellets were then lysed for 10 s with 50 μl lysis buffer (1% NP 40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation at 1600 *g* for 5 min, the supernatant was removed and extraction from the pellet repeated. Apoptotic DNA fragments were recovered from the supernatants by adding SDS to 1% and treating for 2 h with RNase A (final concentration 5 $\mu g^{-1}\mu l$) at 56°C followed by digestion with proteinase K (final concentration 2.5 μg μl) for 2 h at 37°C. DNA was precipitated with 0.5 vol. 10 M ammonium acetate and 2.5 vol. ethanol, dissolved in gel loading buffer and separated by electrophoresis in 1.2% agarose gels. Molecular size markers, both a 100-bp and a λ/*Hin*dIII ladder, were included in the gel for comparison. After gel electrophoresis, the DNA fragments in the gel were stained with ethidium bromide and visualized by UV illumination for ladder formation.

Statistical analysis

All assays were repeated three times to insure reproducibility. The significance of the results obtained from control and treated groups were statistically analyzed by paired Student's *t*-test. Means and standard deviations were calculated. $P < 0.05$ was regarded as statistically significant.

Results

As can be seen in Figure 1, verapamil showed different growth-inhibitory activity against KB cells. When

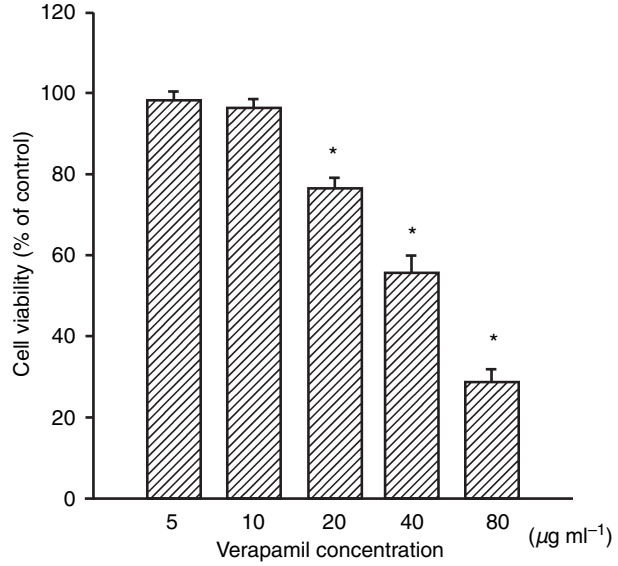

Figure 1 Cytotoxicity assay with MTT staining of viable cells. KB cells were treated with different concentrations of verapamil. Data are shown as the mean of three independent experiments ± s.d. (bars). *Denotes significant differences from control values with $P < 0.05$

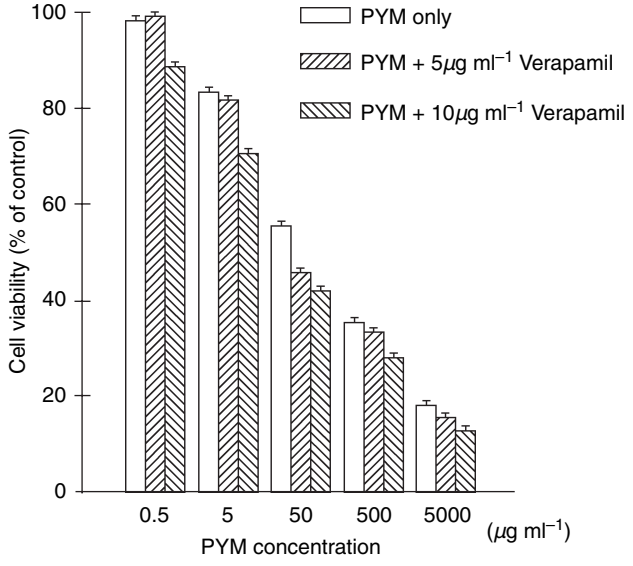

Figure 2 KB cells were treated with different doses of PYM (0.5, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) with or without verapamil (5 or 10 μg ml^{-1}) for 24 h

treated with 20 μg ml^{-1} verapamil, cell viability was significantly inhibited ($P < 0.05$). Therefore, we used non-toxic doses of verapamil (5 or 10 μg ml^{-1}) in our experiments.

The effects of verapamil on the sensitivity of KB cells to PYM are illustrated in Figure 2. Verapamil at non-toxic doses generally enhanced the cytotoxicity of PYM for KB cells. When treated with different doses of PYM (0.5, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}), the viability of KB cells was about 98 ± 1%, 83 ± 1%, 55 ± 2%, 35 ± 2%, and 18 ± 2%. However, the viability of KB cells was decreased to 89 ± 2%, 71 ± 3%, 42 ± 2%, 28 ± 1%, and 13 ± 2% in the presence of 10 μg ml^{-1} verapamil.

The effects of verapamil on the cell cycle of PYM-treated KB cells are presented in Figure 3. As shown by flow cytometry, KB cells in the absence of PYM displayed the normal profile with a peak at 80.8 U of fluorescence representing cells in G_0-G_1 and a peak at 146.3 U of fluorescence representing G_2-M (Figure 3a). Flow cytometric analysis of KB cells treated with 5 mg ml^{-1} PYM revealed a subpopulation displaying propidium iodine fluorescence reduced compared with that of the G_0-G_1 cell cycle region which can be considered as the A_0 region described by Telford *et al* (1991) in cell populations undergoing apoptosis (Figure 3c). On the other hand, compared with Figure 3b, when treated with 50 μg ml^{-1} PYM plus 10 μg ml^{-1} verapamil, the A_0 region was also detected by flow cytometry (Figure 3d).

The results of DNA fragmentation in KB cells with the treatment of various concentrations of PYM with or without verapamil are shown in Figure 4. After incubation for 24 h, DNA ladder bands were detected by the treatment of 500 μg ml^{-1} and 5 mg ml^{-1} PYM without verapamil. No ladder bands were detected when PYM concentrations were below 50 μg ml^{-1}. However, when treated with 50 μg ml^{-1} plus 10 μg ml^{-1} verapamil, DNA ladder bands were clearly observed. The characteristic pattern of DNA fragments consisted of multimers of approximately 180 bp. These digestion products corresponded to the nucleosome ladders characteristic of apoptosis.

Discussion

A series of experiments have demonstrated that the apoptosis of cells is induced by radiation or drugs and the concentration of free intracellular Ca^{++} is found to be markedly elevated in apoptotic cells (Groenendyk *et al*, 2004; Lemarie *et al*, 2004; Sharma and Rohrer, 2004). Verapamil is pharmacologically classified as a calcium channel blocker. It also has other pharmacologic actions, such as reversing multiple-drug resistance for many anticancer agents (Tsuruo *et al*, 1981; Pereira *et al*, 1995), and suppressing metastasis of cancer cells in BALB/c mice injected with mouse mammary adenocarcinoma F3II cells (Farias *et al*, 1998).

In this study, verapamil enhanced the cytotoxicity of PYM in cultured KB cells at non-toxic doses. When treated with 0.5 μg ml^{-1} PYM in the presence of 10 μg ml^{-1} verapamil for 24 h, the viability of KB cells was inhibited significantly. PYM acts directly as an endonuclease and can be considered as an apoptosis mimetic drug at high concentrations (500 μg ml^{-1}, 5 mg ml^{-1}) (Tai *et al*, 2000, 2003). The endonuclease ability of PYM results in two mechanisms of cell death which are closely related to the number of PYM molecules presented inside the cell. By DNA fragmentation analysis, KB cell apoptosis was induced when treated with a lower concentration of PYM (50 μg ml^{-1}) with verapamil (10 μg ml^{-1}) for 24 h. According to

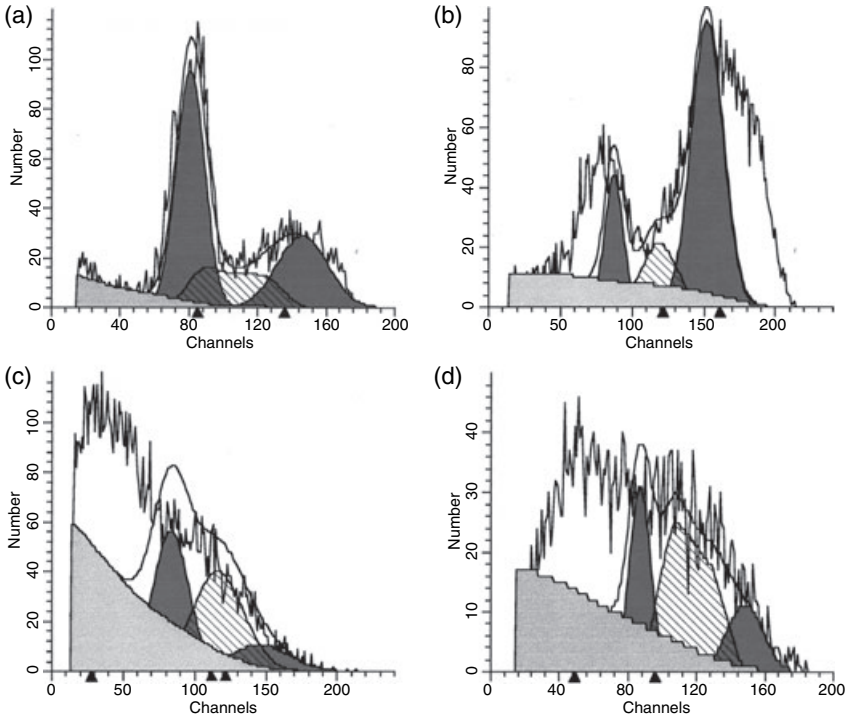

Figure 3 Flow cytometric DNA analysis of KB cells treated with different concentrations of PYM with or without verapamil for 24 h. The number of cells is represented as a function of fluorescence. **(a)** Cells in the absence of PYM display normal profile with a peak at 80.8 U of fluorescence representing cells in G_0-$G_1$and a peak at 146.3 U of fluorescence representing in G_2-M. **(b)** Cells treated with 50 μg ml^{-1} PYM for 24 h. **(c)** Cells treated with 5 mg ml^{-1} for 24 h. Notice the A_0 peak (fluorescence 41.5) characteristic of apoptotic cells. **(d)** In cells treated with 50 μg ml^{-1} PYM and 10 μg ml^{-1} verapamil for 24 h, the A_0 peak (fluorescence 48.3) is also observed

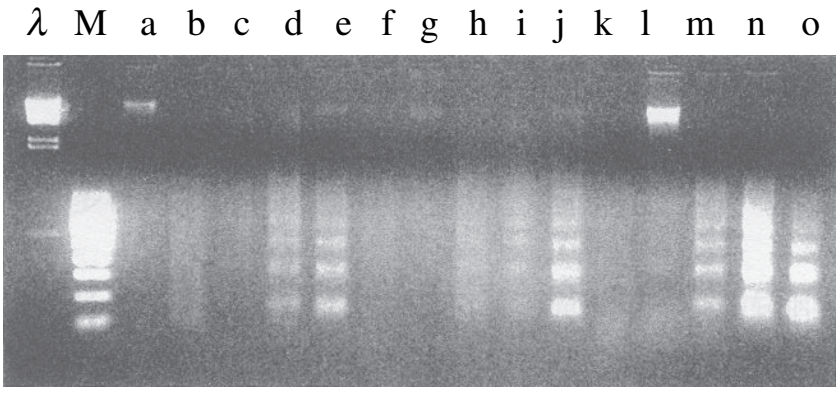

Figure 4 DNA fragmentation in KB cells. (a–e) Cells treated with different concentrations of PYM (0, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) for 24 h. (f–j) Cells treated with different concentrations of PYM (0, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) and 5 μg ml^{-1} verapamil for 24 h. (k–o) Cells treated with different concentrations of PYM (0, 5, 50, 500 μg ml^{-1}, and 5 mg ml^{-1}) and 10 μg ml^{-1} verapamil for 24 h. λ:λ/*Hin*dIII; M: 100 bp ladder

Tsuruo *et al* (1983a), verapamil can efficiently inhibit some antitumor drug efflux function of tumor cells by inhibiting the slow channel of Ca^{++} transport across the cell membrane. The increased intracellular levels of these drugs were directly related to the enhancement of cytotoxicity of the drug (Tsuruo *et al*, 1983b,c). Taken together, the mechanisms of verapamil presumably inhibit PYM efflux from the cells.

Tumor cells having multidrug resistance (MDR) phenotype exhibit a reduced sensitivity to cytotoxic drugs when compared with the parental drug-sensitive cells. The cellular pharmacologic basis for MDR *in vitro* appears to be a reduced steady-state accumulation of drugs in resistant cells compared with sensitive cells, caused by the overexpression of the *mdr1*gene which encodes for a P-glycoprotein of 170–180 kDa. P-glycoprotein is thought to act as an energy-dependent drug efflux pump (Bradley *et al*, 1988; Gottesman and Pastan, 1993). Verapamil has been shown to increase the sensitivity of MDR cells to drugs, thus reverting drug resistance (Tsuruo *et al*, 1981, 1983b,c). Thus, the mechanism responsible for the MDR of verapamil may be explained as follows. Verapamil enhances antitumor agent retention through competition for closely related binding sites on P-glycoprotein (Yusa and Tsuruo, 1989). Verapamil is also actively transported outward. These findings suggest that verapamil reverses MDR by competitively inhibiting drug transport. P-glycoprotein could recognize verapamil as substrate as well as an antitumor agent (Kessel, 1986; Cano-Gauci and Riordan, 1987). Taken together, verapamil is therefore of potential therapeutic use.

From a clinical viewpoint, our present experiments indicate that verapamil may enhance the cytotoxicity of PYM in chemotherapy for the treatment of malignant tumors. Verapamil might have important implications in the reversion of MDR in tumor clinical work. On the other hand, it might be very beneficial to induce tumor cell apoptosis in cancer chemotherapy. Necrotic cell

death would release intracellular constituents into tissue upon cell destruction. Therefore, if we could induce tumor cell apoptosis in the treatment of malignant tumor, it would prevent secondary tissue damage due to inflammatory reaction caused by necrotic cell death (Fernandes and Cotter, 1994). In our study, verapamil was found to promote PYM responsiveness to induce tumor cell apoptosis *in vitro*. Our earlier result indicated that in order to induce cell apoptosis, the concentration of PYM (500 mg ml^{-1}, 5 mg ml^{-1}) present in the culture medium is too high for clinical use (Tai *et al*, 2000). Therefore, verapamil, a membrane-modifying agent affects the transport function of PYM through the plasma membrane, resulting in inducing apoptosis of tumor cells at lower concentrations of PYM might resolve this problem.

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